Optimization of PMA-PCR Protocol for Viability Detection of Pathogens

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In the event of a bioterrorism attack or epidemiologic outbreak, the ability to efficiently determine whether an area is safe, as well as what species are present, is essential. This project's objective was to optimize protocols for use in creating a portable instrument capable of determining viability and identification simultaneously. This is made possible by the use of the DNA-intercalating agent propidium monoazide (PMA) combined with molecular biology analyses including polymerase chain reaction (PCR). PMA selectively binds to dead cells, inhibiting polymerase activity, making DNA unable to be amplified by PCR. By inhibiting the amplification of DNA of any dead cells, and using specific primers, PMA-PCR results consist purely of viable samples of the target species. PMA-PCR protocol was optimized and the correlation between viable to dead cell ratio with PMA-PCR output was determined using methods of DNA extraction, PCR, phase-contrast microscopy, and cell culture. Viability indicators as measured by these methods varied among different inactivation treatments, which included heat-kill, UV irradiation, and autoclaving. Loss of culturability by heat-kill treatments left DNA intact, resulting in amplification of PCR products, whereas, UV irradiation may have degraded DNA such that there was a decrease in all measurements (PCR, CFUs, and PMA-PCR). This presented study demonstrates the need that PMA-PCR can be used to capture the loss of viability of a sample that is much more specific and time-efficient than alternative methods. This protocol is particularly useful in scenarios in which sterilization treatments may inactivate organisms but not degrade their DNA. The use of a PCR-based method of pathogen detection without first inactivating the DNA of nonviable cells will potentially lead to false positives. The loss of culturability, by heat-killing, did not prevent amplified PCR products, which supports the use of PMA to prevent amplification and differentiate between viable and dead cells. PMA was shown to inhibit the amplification of DNA by PCR in vegetative cells that had been heat-killed.

I. Introduction

In the event of a bioterrorism attack or a possible epidemiologic outbreak, the ability to determine whether an area is safe as well as what species is present in the shortest amount of time is essential. This assessment is often achieved through culture based methods. However, these methods are often time consuming and leave a wider margin for error. For example, culture-based methods for measuring cells or viruses can take hours or longer to analyze. Another method, involving ATP analysis, provides only an estimate of the amount of viable biomass in a sample and is not selective for a specific pathogen unless coupled with antibodies or some other selection process.[1] Other viable microorganisms in the sample could produce false positives or inaccurate results. Lastly, a LIVE/DEAD® viability assay, which uses a combination of a membrane-permeant and a membrane-impermeant

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fluorescent dye, is able to determine the viability of the sample.[2] As with ATP analysis, this technique also lacks the ability to select for a specific pathogen. PCR is another potential method that is highly specific; however, it may detect the DNA of both viable and non-viable organisms[3].

The need for a portable instrument, capable of determining viability and identification within the same protocol, is immense. This process is made possible by the use of the DNA-intercalating agent propidium monoazide (PMA) combined with molecular biology analyses such as PCR or qPCR.[4] According to the manufacturer, Biotium, PMA is cell membrane-impermeable, so it will selectively bind to the DNA of dead cells, whose cell membranes have degraded. PMA is able to be photochemically linked to DNA by a stable nitrogen-carbon bond.[5] This covalent modification to DNA inhibits polymerase activity, so the DNA from non-viable/membrane compromised cells is unable to be amplified by PCR. By inhibiting the amplification of DNA of dead cells, PCR results consist purely of viable samples. Specific primers will also be used to selectively amplify only a specific species' DNA. This combination of a viability assay and identification technique thus enables the determination of viability on-site, in a much shorter time-period, for a specific pathogen in question.

The primary objective of this project is to optimize and test various PMA protocols with pure cultures of pathogen surrogates, which will ultimately be used to inform the development of a portable instrument. This objective will be accomplished by preparing a stock of test organisms, optimizing PCR and PMA protocols, and evaluating enumeration methods conducted on a set of experiment in which organisms are inactivated by different processes (heat-killed, UV irradiation, and autoclaving).[5] The enumeration techniques that will be evaluated include optimized PCR and PMA-PCR as well as culture-based methods.

II. Materials and Methods

A. Obtaining *B. cereus* cell suspensions (pure spore, vegetative cells).

A stock of test cultures of *Bacillus cereus* endospores was created using *Bacillus* Sporulation Protocol (Mesophile).[6] Vegetative cells were obtained by plating a *B. cereus* endospore suspension on Tryptic Soy Agar (TSA) plates and incubated at 30°C for 24-48 hours. Sporulation was then induced by inoculating cells to sporulation media. The plates were incubated at suboptimal temperature (30°C) for 3-7 days to induce sporulation. Samples were determined to contain >80% free endospores by using a phase contrast microscope. The samples were collected from 5 plates and placed in a centrifuge tube. After the cells were washed and centrifuged according to protocol, remaining vegetative cells and debris were degraded with lysozyme and washed until >99.99% endospores remained. Spore suspensions were verified using phase-contrast microscopy. To obtain vegetative cells, endospores were transferred to fresh media, nutrient broth or TSA plates, and incubated at 37°C for 24-48 hours.

B. Measurement of Culture Samples

Samples were serially diluted, if necessary, to obtain a countable measure of 25-250 CFUs per plate. 100 µl of sample was added to a TSA plate and spread using a sterile spreader. TSA plates were incubated at 37°C for 24-48 hours for quantification of colony forming units (CFU) per 100 µl.

C. DNA Extraction

DNA extraction was performed on *B. cereus* endospores and vegetative cells using MoBio PowerLyzer™ PowerSoil DNA Isolation Kit (MoBio, Carlsbad, CA). 100 µl of sample was added to glass-bead beating tubes. The DNA was eluted in 100 µl of C6 elution buffer (provided). Because of the time-intensive nature of the MoBio protocol, an alternate protocol was adapted from EPA Method B. In this protocol, samples underwent a crude extraction by which 100 µl of sample was added to PowerLyzer™ Glass Bead Tubes, 0.1mm (MoBio, Carlsbad, CA). 300 µl of AE Buffer [10 mM Tris-Cl, 0.5 mM EDTA, pH 9.0, used in DNeasy kits (Qiagen, Valencia, CA)] was added and cells were lysed using a Vortex-Genie® 2 Vortex adapter (MoBio, Carlsbad, CA) on maximum speed for 10 minutes. Samples were pelleted by centrifugation at 10,000 x g for 30s. The supernatant product was removed and stored at -30°C for further analysis.

D. PCR Amplification

25 µl samples were prepared for PCR by adding 1 µl of DNA extract to 24 µl of a master mix. The master mix was created using 12.5 µl of MyTaq™ Red Mix 2x (Bioline, Tauton, MA), 1 µl forward primer (10 µM stock), 1 µl reverse primer (10 µM stock), and 9.5 µl molecular grade H_2O. PCR was performed using a Bio-Rad iCycler 1.280 (BioRad, Hercules, CA). Thermal cycles were 94°C for 15 s, 50°C for 10 s, and 74°C for 35 s, for 37 cycles. PCR products were then stored at -20C. The primers used for targeting the *B. cereus* hemolysin gene were derived from

Wang et al.[7] BC-1 (Forward): 5'-CTGTAGCGAATCGTACGTATC-3' and BC-2 (Reverse): 5'-TACTGCTCCAGCCACATTAC-3'

E. Gel Electrophoresis

Electrophoresis was performed on amplified PCR products using a LIBERTY1 gel system (Biokeystone, Portland, OR). The 1% agarose gel was prepared by adding 1.0 g of agarose to 100 mL of 0.5% TAE buffer and microwaved until fully dissolved. 7.5 µl of ethidium bromide was added to the 1% agarose solution. 8 µl of PCR products from each sample were visualized on the gel alongside 5 µl of MassRuler™ Low Range DNA Ladder (Thermo Fisher Scientific, Glen Burnie, MD). Electrophoresis was run at 100V for 45 minutes. The gel was examined using a UVP Epichemi[3] Darkroom (UVP, Upland, CA) under UV-light (365nm) and analyzed using LabWorks software (UVP, Upland, CA).

F. Differential Kill Methods

B. cereus endospores and vegetative cells were subjected to variable kill methods to produce samples with 0% viable cells as measured by cultured-based methods.

Heat–killed endospores and vegetative cells were produced by adding 0.5-1.0 mL of sample containing approximately 10^5 CFUs per 100 µl to a 2.0 mL screw-cap microcentrifuge tube. Tubes were heated using a heat block for 30 minutes at 95°C. Negative controls were established by adding 1.0 mL of 18.2 MΩ filter-sterilized H_2O to a 2.0 mL tube. One tube was heated at 95°C for 30 min and one left at 25°C for 30 min. Positive controls consisted of 3 tubes at 25°C for 0 minutes and 30 minutes.

UV-irradiated *B. cereus* endospores and vegetative cells were produced by adding 0.5-1.0 mL of sample containing approximately 10^5 CFUs per 100 µl to a 2.0 mL screw-cap microcentrifuge tube. Samples were exposed to germicidal UV-light (254 nm) for 30 min at a distance of 8 cm. A negative control was established by adding 1.0 mL of 18.2 MΩ filter-sterilized H_2O to a 2.0 mL tube and exposed to UV (254nm) for 30 min. The same positive controls were used for both heat-killed and UV-killed cell trials.

Decrease/change in viability was assessed using culture techniques; essentially, 100 µl of killed samples were plated onto TSA plates and incubated at 37°C for 24-48 hours to verify lack of culturable cells.

G. PMA Treatment

The propidium monoazide (phenanthridium, 3-amino-8azido-5-[3-diethylmethylammonio]propyl)-6-phenyl dichloride) treatment of cells was adapted from Rawsthorne et al.[3] A 500 µl sample was brought to a working concentration of 10 µM by adding 2.5 µl of 2 mM PMA stock solution containing 20% DMSO using clear plastic 1.0 mL screw-cap microfuge tubes. A negative control was established by adding 1.0 mL of 18.2 MΩ filter-sterilized H_2O to a 1.0 mL tube. The tubes were incubated in the dark for 50 min. The tubes were then exposed to light for 3.0 min using a 500W halogen lamp at a distance of 12 cm. The microfuge tubes were floated horizontally on a water/ice mix to lessen the number of cells killed due to overheating. Samples were then plated to verify culturability and DNA extracted for use in PCR. For a subset of spore samples, dithiothreitol (DTT) was also used to disrupt the outer coat.

III. Results & Discussion

A. PCR & Detection Level Optimization

Detection of samples of *B. cereus* was improved to as low as 580 CFUs by optimizing extraction and PCR protocols. Initially only samples containing ~10^4 cells were detectable using our original protocols. It was discovered that glass bead beating tubes were much more efficient at cell/endospore lysis than the large garnet bead tubes (obtained from the UltraClean MoBio Kit). Large quantities of primer dimers were observed and partially inhibited the amplification of DNA the samples as in Fig 1. This further reduced the ability to detect samples containing lower numbers of CFUs. To address this issue, we used various ratios of forward and reverse primers and also evaluated the effect of keeping the mastermix on ice. The resulting gel showed that the best detection of spores was found using the original 1:1 ratio of forward:reverse primers, at 1 µl each, and with master-mix on ice.

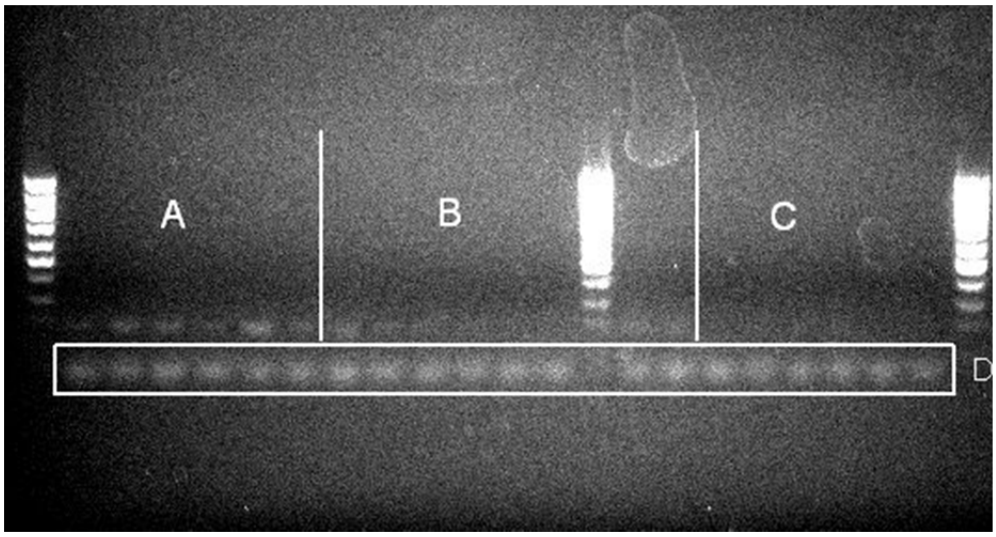


Figure 1. Electrophoresis gel of *B. cereus* endospores. *Wells contain PCR products from samples containing A) 1,450 CFUs, B) 580 CFUs, and C) 290 CFUs. Primer dimers were observed in box labeled "D"*

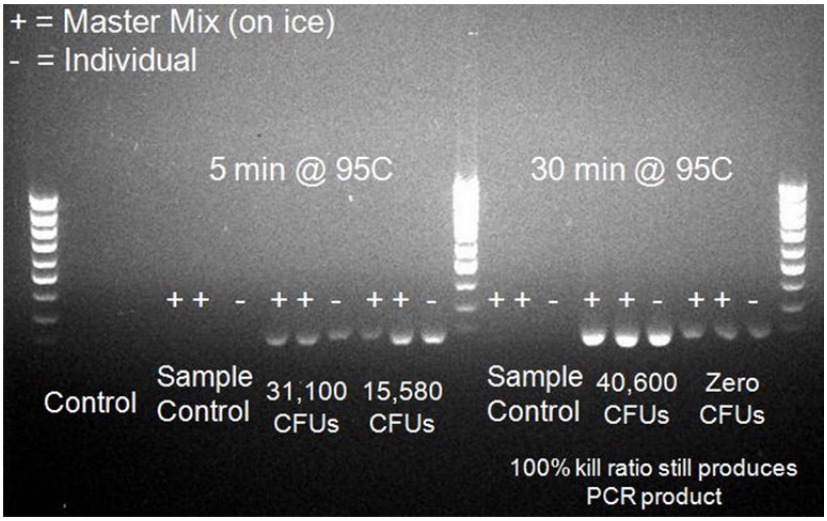


Figure 2. Electrophoresis gel of heat killed *B. cereus* endospores. *Nonviable (unculturable) endospores were shown to still produce a significant amount of PCR products. Keeping master mix on ice prevented the appearance of primer dimers.*

B. Differential Kill Methods

We subjected samples containing ~10^5 endospores or vegetative cells per 100 µl to differential methods of cell-death. These were a heat-block at 95°C for 30 minutes, germicidal UV-light (254 nm) at 8 cm for 30 minutes, and autoclave (121°C, 15 PSI, 15 minutes). Positive controls were incubated for each treatment for 0 min, kept at 4°C, and 30 min at room temperature. The autoclave and heat-block succeeded in killing 100% of the spores and vegetative cells. Vegetative cells did not survive UV-treatment as measured by culture-based methods but some endospores remained culturable. We observed that the autoclave eliminated both amplifiable DNA as well as culturable organisms. While the heat-block treated cells of both endospores and vegetative cells were completely non-culturable, a significant amount of PCR products were still observed in Figs 2 & 3. As expected, the UV-light exposure greatly reduced the amount of PCR products in vegetative cells by damaging the DNA, as in Fig 3. However, this was not the same for endospores. While the endospores had a greatly reduced culturability, the amount of PCR products observed remained high. Since the heat-killed cells, and the UV-killed to some degree, had significant PCR products, this supports the need to use a reagent such as PMA to differentiate between living and dead cells before PCR is performed. However, the use of PMA to differentiate between viable and dead cells will be dependent on the optimal use of sterilization techniques and the application of this methodology.

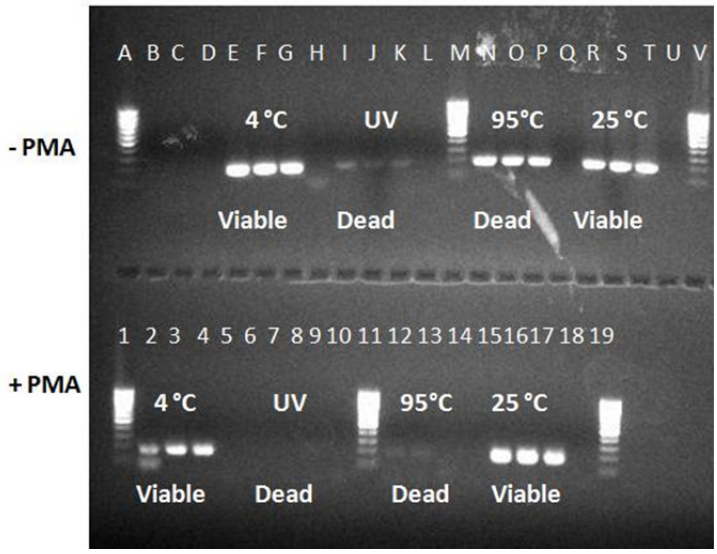
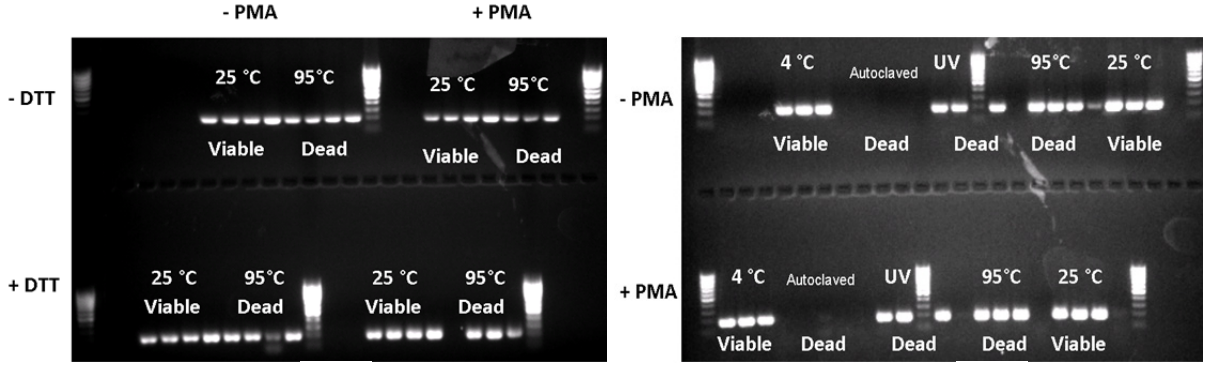


Figure 3. Electrophoresis gel of PMA treated *B. cereus* vegetative cells. *Vegetative cells were killed using heat-block at 95°C for 30 minutes and UV-light (254 nm) for 30 min. Positive controls were left at 4°C and 25°C for 30 min. PMA was shown to greatly inhibit the amplification of DNA in nonviable cells.*

C. *B. cereus* Vegetative-PMA Experiment

We subjected samples of 10^5 cells to the kill methods of heat-block at 95°C for 30 min and germicidal UV-light (254 nm) at 8 cm for 30 min to produce our 100% killed samples. These were plated and verified to have zero CFUs. Positive controls were made using 10^5 CFU samples and keeping one set at 4C and one set at 25°C for 30 min. Negative controls for each test group consisted of filter-sterilized H_2O. The vegetative cells that were not treated with PMA showed significant PCR products for both sets of living cells and heat-killed cells. The UV-killed cells showed a reduced amount of PCR product but bands were still observed.

When these samples were treated with PMA, the viable control samples showed an unchanged intensity of PCR product. However, both the heat-killed and UV-killed sample band intensities were greatly reduced. The wavelength needed for PMA to covalently link to DNA is 464 nm, which falls within the blue-range of visible light. [8] A blue LED may be preferable to use in the future as the intensity of heat & light emitted by the 500W halogen bulb killed a significant number of cells, approximately 50-60% (Data not shown).[9]



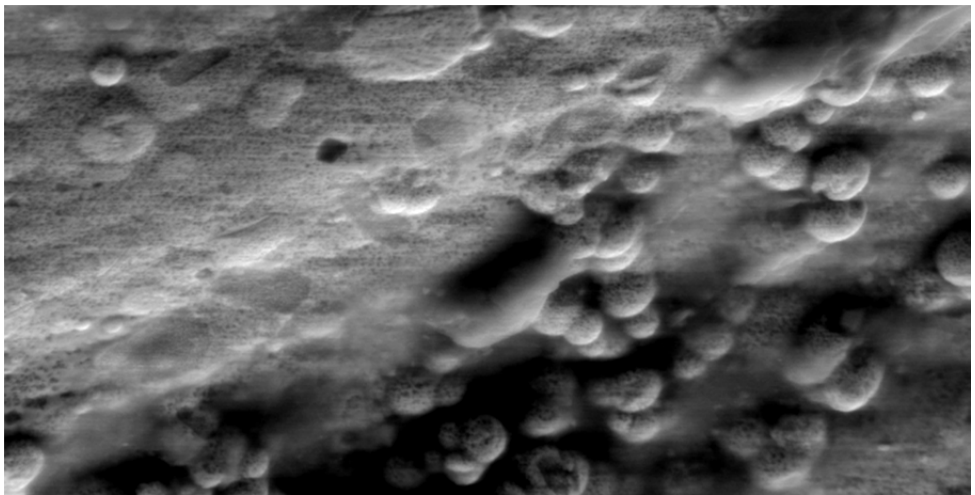
a) b)

Figure 4a. Electrophoresis gel of PMA and/or DTT treated *B. cereus* endospores. *Using samples of 100% viable and 100% unculturable cells, PMA treatment was unable to penetrate the spore coat in order to bind with DNA and inhibit PCR. DTT was used in an attempt to facilitate the entry of PMA into the cells. We observed no significant difference in band intensity from cells treated with or without PMA/DTT.*

Figure 4b. Differential methods treated with PMA. *Cells killed by using autoclave, heat-block, and UV-irradiation, were subjected to PMA treatment. We observed no difference in the amount of PCR products amplified.*

D. *B. cereus* Endospore-PMA Experiment

We subjected endospores to the same conditions as vegetative cells and observed that both 100% viable and 100% unculturable samples exhibited the same amount of band intensity when amplified by PCR (Figure 4). When these samples were treated with PMA, there was no observable difference in band intensity. We hypothesized this was due to the inability of PMA to penetrate the spore coat. We attempted to facilitate the permeability of PMA through the spore coat by first treating the endospores with dithiolthreitol (DTT). We did not observe any difference in band intensity when endospores were treated with DTT & PMA or PMA alone.



E. Electron Microscopy

Further evidence to our hypothesis that an intact spore coat is inhibiting the permeability of PMA was seen under environmental scanning electron microscopy. When samples of 100% unculturable heat-killed endospores were viewed at 1604x, it was clearly visible that not all endospores had visibly damaged spore coats. A significant number of them remain intact, even though they are nonviable. This prevents PMA from permeating into the cell and intercalating into the DNA. Without the ability to get the PMA inside the spore, differentiation is not possible. Further methods of allowing PMA entry to dead cells must be tried in the future.

IV. Conclusion

The presented work shows that PMA was able to inhibit the amplification of DNA by PCR. These observations highly support the use of PMA to differentiate between living and dead vegetative cells that is also highly species-specific. Further work is required to enable PMA pretreatment to reliably differentiate between living and dead endospores. This pretreatment may be improved by facilitating non-viable cell entry of PMA by decoating spores or germinating the endospores first prior to incubation with PMA. By using standard PCR, we were able to validate the functionality of PMA only qualitatively. Going forward, the use of quantitative PCR (qPCR) is highly suggested to determine the change in cycle threshold (Ct) values for samples treated with and without PMA and also to improve the detection limit. Furthermore, we found that even short periods of exposure to a halogen lamp may reduce cell viability, as measured by culture-based methods; a blue LED may be a more appropriate light source that can efficiently activate cross-linking of PMA to DNA, while preserving the number of viable cells.

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